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A FRIZZLED QUEST TO DISSECT THE MOLECULAR PHARMACOLOGY OF WNT SIGNALING:

FROM BIOLOGY TO SIGNALING MECHANISM(S)

Elisa Arthofer

Stockholm 2017

Cover: Abstract modification of images of HEK293 cells transfected with fluorescently-tagged FZD_4, $G\alpha_{12}$, and p115-RhoGEF. Original images by Belma Hot, creative abstraction by Lucia Arthofer. All right reserved.

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A FRIZZLED QUEST TO DISSECT THE MOLECULAR PHARMACOLOGY OF WNT SIGNALING: FROM BIOLOGY TO SIGNALING MECHANISM(S)

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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"Here is a biologist examining a culture of nerve cells in a small dish. One set of nerve cells examining another set of nerve cells. Not quite a trivial scenario."

Anonymous

For Panna

ABSTRACT

The wingless/int1 (WNT)/Frizzled (FZD) family of signal transduction pathways is highly conserved across species and controls essential physiological functions important for embryonic development, stem cell renewal, proliferation, differentiation, and cell polarity. Dysregulation of these signaling pathways leads to developmental abnormalities or other conditions such as inflammation, cancer, or neurological disorders. In mammals, 19 different WNTs can bind to and interact with ten isoforms of FZD in a plethora of combinations. These seven transmembrane-spanning receptors are categorized in the Class Frizzled within the superfamily of G protein-coupled receptors (GPCRs). Several important co-factors are known to aid in the activation of WNT/FZD signaling, such as Disheveled (DVL) or low density lipoprotein receptor related protein 5 and 6 (LRP5/6). In addition, interactions of FZDs with heterotrimeric G proteins have continuously been reported. Upon ligand binding, activation of β-catenin-dependent and/or β-catenin-independent downstream signaling pathways takes place.

The overall aim of this thesis was to shed light on mechanistics of WNT/FZD signaling and pharmacology from different angles: In paper I, we investigated the presence and role of WNT-5A in human glioblastomas, a WNT important for neurological functions in the central nervous system (CNS) and found to be dysregulated in many cancers. In this study, we describe the correlative nature of high WNT-5A expression with upregulation of genes involved in immunological processes as well as increased microglia infiltration in the tumor microenvironment. In paper II and III, we focus on FZD_4, a FZD isoform important for retinal vascularization. We provide functional evidence for the interaction of FZD_4 with heterotrimeric $G\alpha_{12/13}$, which is independent of DVL and LRP5/6, and show activation of downstream signaling events. We further describe a novel signaling route through Norrin-FZD_4-$G\alpha_{12/13}$, which exerts an inhibitory effect on the classical Norrin-FZD_4-β-catenin signaling pathway known to be important in angiogenesis, thus arguing for a concept of cross-talk and feedback inhibition from the same FZD isoform, a notion that is as of yet completely unappreciated.

In addition, this thesis tries to point out the current limitations and struggles in the field of studying WNT/FZD signaling and the need for further studies identifying crucial links to signal specification, which would aid in future drug development targeting this pathway.

LIST OF SCIENTIFIC PAPERS

I. **High levels of WNT-5A in human glioma correlate with increased presence of tumor-associated microglia/monocytes**

Dijksterhuis J, **Arthofer E,** Marinescu V, Nelander S, Uhlén M, Pontén F, Mulder J, Schulte G. Experimental Cell Research. 2015 Dec;339(2):280-8

II. **WNT stimulation dissociates a Frizzled 4 inactive state complex with $G\alpha_{12/13}$**

Arthofer E*, Hot B*, Petersen J, Strakova K, Jäger S, Grundmann M, Kostenis E, Gutkind JS, Schulte G. Molecular Pharmacology. 2016 Oct 1;90 (4) 447-459; Selected as the cover of the October 2016 issue.

III. **$G\alpha_{12/13}$ as a central regulator of FZD_4 signaling**

Arthofer E, Zhang C, Junge H, Gutkind JS, Balla T, Schulte G *Manuscript*

* These authors contributed equally.

ADDITIONAL CONTRIBUTIONS NOT INCLUDED IN THIS THESIS

I. FZD_{10}-$G\alpha_{13}$ signalling axis points to a role of FZD_{10} in CNS angiogenesis

Hot B, Valnohova J, **Arthofer E,** Simon K, Shin J, Uhlén M, Kostenis E, Mulder J, Schulte G. Cellular Signaling. 2017 Jan 24;32:93-103.

II. The Concise Guide to PHARMACOLOGY 2013/14: G protein-coupled receptors

CGTP Collaborators. British Journal of Pharmacology. 2013 170(8):1449-58.

III. Class Frizzled GPCRs entry for the IUPHAR database

Contributor. http://www.guidetopharamcology.org

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LIST OF ABBREVIATIONS

7-TM	Seven-transmembrane
APC	Adenomatous polyposis coli
cAMP	Cyclic adenosine monophosphate
CK1	Casein kinase 1
CL	Cross-linking
CNS	Central nervous system
CRD	Cysteine rich domain
DAVID	Database for Annotation, Visualization and Integrated Discovery
dcFRAP	Dual-color fluorescence recovery after photobleaching
DKK-1	Dickkopf-1
DMR	Dynamic mass redistribution
DNA	Deoxyribonucleic acid
DVL	Disheveled
EMT	Endothelial to mesenchymal transition
ERK	Extracellular signal-regulated kinase
FEVR	Familial exudative vitreoretinopathy
FRET	Förster resonance energy transfer
FRAP	Fluorescence recovery after photobleaching
FZD	Frizzled
GAP	GTPase-activating protein
GBM	Glioblastoma multiforme
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GSEA	Gene set enrichment analysis
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
GTPγS	Non-hydrolysable G protein activating analog of GTP
HEK293	Human embryonic kidney cells 293
HeLa	Commercial cell line named after Henrietta Lacks
HEPG2	Commercial hepatocellular carcinoma cell line

HLA	Human leukocyte antigen
HLA-DMA	Major histocompatibility complex, Class II, DM alpha
HLA-DPB1	Major histocompatibility complex, Class II, DP beta 1
IBA-1	Ionized calcium-binding adapter molecule 1
IP_3	Inositol triphosphate
IUPHAR	International Union of Basic and Clinical Pharmacology
IWP	Inhibitor of WNT production
JNK	c-Jun N-terminal kinase
LRP5/6	Low density lipoprotein receptor related protein 5 and 6
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NFAT	Nuclear factor of activated T cells
PCP	Planar cell polarity
PDZ	Postsynaptic density 95/disc-large/zona occludens-1
PORCN	Porcupine
PTX	Pertussis toxin (from Bordetella pertussis)
RAC	Ras-related C3 botulinum toxin substrate 1
RGS	Regulator of G protein signaling
RHOA	Ras homolog gene family, member A
RhoGEF	RhoGTPase guanine nucleotide exchange factor
RNA	Ribonucleic acid
ROR1/2	Receptor tyrosine kinase-like orphan receptor 1 and 2
RYK	Related to receptor tyrosine kinase
SMO	Smoothened
TCF/LEF	T-cell specific transcription factor/Lymphoid enhancer factor
TCGA	The cancer genome atlas
Wg	Wingless
WHO	World Health Organization
WNT	Wingless/int-1
YAP	Yes-associated protein 1
TAZ	Transcriptional co-activator with PDZ-binding motif

1 INTRODUCTION

1.1 THE BEGINNING OF A NEW ERA

Cellular signaling, or also commonly referred to as signal transduction, is the process of cells communicating with their environment or with each other, and subsequently responding to external cues being sensed during that process. This phenomenon of cells temporally sensing and responding to external stimuli basically represents one of or "the" essence(s) of life itself, in its smallest entity.

Despite many cells having somewhat similar capacities to sense and react to surrounding stimuli of all sorts, there can be a great variation in response, purpose, and mechanism. Yet, the extent of similarity across species is remarkable, especially for example in the eukaryotic kingdom, where comparative studies across species can be applied as a tool to advance the field of cell signaling in humans.

The first mentioning of scientific concepts somewhat related to cell signaling can be traced back as early as 1855, where French physiologist Claude Bernard studied the human pancreas and liver and described a then novel concept of "internal secretion", which would later be known as the endocrine system. Despite describing glucose and its precursor in the liver, glycogen, in astonishing detail for that time, it wasn't until early 1900 that two scientists named Sir William M. Bayliss and Ernest H. Starling would discover the chemical messenger secretin, later named the first "hormone", while studying the process of digestion in dogs (Bayliss et al., 1902). Starling introduced the term hormone (derived from the Greek meaning "to arouse or excite") first in 1905 as part of his studies on chemical control of physiological functions in the body. He insisted that hormones are "…the chemical messengers which speeding from cell to cell along the blood stream, may coordinate the activities and growth of different parts of the body…" (Starling, 1905). Parallel studies by George Oliver and Sir Edward A. Sharpey-Schäfer on suprarenal glands led to the early discovery of adrenaline as well as setting the foundations for the field

of endocrinology (Oliver et al., 1894). And if one will, those early and primitive yet not trivial studies on internal secretions, namely the discovery of secretin as well as adrenaline, might have paved the way for a field in science now commonly known to us as cell signaling.

The idea for receptors as we know them today originated in the early 1900's. On one hand, John Langley performed a multitude of experiments on sympathetic neuroeffector transmission challenging the then popular notion that drugs would act at nerve endings only (Langley 1901, 1905). On the other hand and around the same time as Langley, Paul Ehrlich laid out some of the basics for a receptor concept in his studies on toxins and his "receptive side chain theory", from which he would later derive the term "receptor", comparing it in the context of cellular toxins to a "lock and a key" (Ehrlich, 1956). Ehrlich was awarded the Nobel Prize for Physiology or Medicine in 1908 together with Ilya Metchnikoff for their contributions to the field of immunology (""The Nobel Prize in Physiology or Medicine 1908"," 1908).

The earliest structural models for G protein-coupled receptors (GPCRs) were derived from correlations with a bacterial integral membrane protein, the bacteriorhodopsin, for which structures had been resolved almost a century after Ehrlich's initial receptor theory (Grigorieff et al., 1996; Kimura et al., 1997; Pebay-Peyroula et al., 1997). Only several years later, the first crystal structure of a mammalian GPCR was solved with experiments on the bovine rhodopsin receptor (Palczewski et al., 2000). In 2007, the first crystal structure of a human GPCR, the β_2-adrenergic receptor, followed (Rasmussen et al., 2007) and a high-resolution model was constructed (Cherezov et al., 2007; Rosenbaum et al., 2007). Since then, close to 800 members of the superfamily of GPCRs have been identified (Bjarnadottir et al., 2006), and solving the crystal structure of each and every one of them remains a major challenge in the field, with many orphan GPCRs not even having ligands identified as of yet.

1.2 WNT/FRIZZLED SIGNALING

1.2.1 A brief history of WNTs and Class Frizzled receptors

The definition of the WNT family of lipoglycoproteins was based on two historical findings several decades ago. On one hand, Varmus and Nusse, who had worked on identifying oncogenes in mice causing mammary tumors, identified in 1982 a new proto-oncogene with the ability to transform mouse mammary cells, which they termed *int-1* (derived from "integration" of the MMTV virus into the genome of mouse mammary carcinomas) *(Nusse et al., 1982)*. Slightly earlier, studies on embryonic development in fruit flies (*Drosophila melanogaster)* led to the discovery of a gene causing deformation and loss of wing tissue when mutated, and was thus termed *wingless (Wg) (Sharma, 1973)*. It took several more years for scientist to realize that the mouse *int-1* gene and the drosophila *wingless* gene were homologues (Cabrera et al., 1987; Rijsewijk et al., 1987). Once further *int-1*-related genes were discovered but in different manners, the scientific community agreed to rename these genes, which showed prospects of becoming a substantial new family of mammalian genes, into Wnt, a mnemonic for *int* and *Wg* (Nusse et al., 1991). The discovery of the WNT family indeed opened up an immense field of research enabling insights into the details of molecular signaling in development, physiology, as well as diseases and many new and promising pharmacological targets (reviewed extensively in (Klaus et al., 2008)).

FZDs, the receptors for WNTs, were identified around the same time as their ligands but independently of them, precisely through a screen for mutations in *Drosophila* polarity genes (Bridges et al., 1944). The fruit fly provides an excellent system for studying embryonic development, and in particular cell and tissue polarity. And so, the *frizzled* pathway soon became a popular subject for studying planar polarity in different body regions of the fruit fly, such as the wings, the eyes, as well as sensory bristles (Gubb et al., 1982; Vinson et al., 1987; Wong et al., 1993).

The connection, that WNTs are the ligands for FZDs, was made several years after these proteins were first discovered. It was shown that in *Drosophila*, mutations in *frizzled* and *disheveled*, later to be identified as a crucial scaffold protein in the WNT/FZD pathway, caused a similar phenotype in vivo (Krasnow et al., 1995). Shortly after, more evidence was gathered strengthening the idea and finally confirming that FZDs function as receptors for WNTs (Bhanot et al., 1996; Yanshu Wang et al., 1996).

1.2.2 WNTs and Norrin

WNTs in mammals constitute a group of 19 different lipoglycoproteins, all of which are by now commercially available as recombinant proteins (R&D, USA). Purification of WNTs has been a challenge ever since it was first achieved due to its lipid modifications (Willert et al., 2003). WNTs are purified by harvesting conditioned media of WNT-overexpressing mammalian cells followed by fractionation, immobilization, and subsequent purification (Schulte et al., 2005; Willert et al., 2003). Despite many modifications to this process, it remains a challenge to obtain intact, lipophilic and biologically active WNT protein and in addition, the use of conditioned media introduces a range of additional and sometimes unknown factors into experimental setups.

Norrin was discovered to be an atypical, FZD_4-selective ligand after studies in mice with mutations in FZD_4 showed compelling similarities to mice displaying Norrie disease, an eye disorder caused by defective retinal vascular development (Berger et al., 1996). Earliest report on the Norrin-FZD_4 interaction showed that together with the co-receptor LRP5/6, Norrin and FZD_4 activate WNT/β-catenin signaling in mammalian cells (Xu et al., 2004). Besides its angiogenic properties important for vascularization of the eye and the inner ear (Luhmann et al., 2005a; Ohlmann et al., 2005; Rehm et al., 2002; Richter et al., 1998), Norrin has also been shown to exert neuroprotective properties via the WNT/β-catenin signaling pathway (Seitz et al., 2010). In addition, loss of

Norrin in mice affects reproduction through loss of vascularization and malformation of the endometrium (Luhmann et al., 2005b).

1.2.3 Class Frizzled receptors

FZDs share typical structural features with GPCRs, most commonly the well-known 7-transmembrane-spanning structure, but also lack substantial other core features like important conserved structural domains, and have thus been categorized per the International Union of Basic and Clinical Pharmacology (IUPHAR) classification as class Frizzled receptors within the GPCR receptor superfamily. This class contains 10 mammalian FZDs and one Smoothened (SMO) receptor (Foord et al., 2005). Amongst themselves, FZDs show various degrees of sequence homologies and several clusters can be differentiated: $FZD_{1, 2, 7}$ (75%), $FZD_{5, 8}$ (70%), $FZD_{4, 9, 10}$ and $FZD_{3, 6}$ (50%) (Fredriksson et al., 2003). Besides the 7-TM structure, FZDs contain an extracellular N-terminus with a signal sequence followed by a cysteine-rich domain (CRD), the putative site for ligand recognition and binding of some co-receptors (Xu et al., 1998). The transmembrane core of the receptor consists of 3 intracellular and 3 extracellular loops as well as an intracellular C-terminus of various lengths. The C-terminus is characterized by a conserved KTxxxW domain for binding the PDZ domain of the scaffold protein Disheveled (DVL) (Umbhauer et al., 2000), several potential contact sites for heterotrimeric G proteins, as well as a terminal domain for binding of other PDZ-domain proteins (Schulte et al., 2007). In addition, a wide range of different phosphorylation sites has been identified for most FZDs, most of them though based on in silico analyses of conserved structural features (Schulte, 2010), rather than *in vivo/vitro* data (Yanfeng et al., 2006).

1.2.3.1 Disheveled

One major interacting partner of FZDs is the cytoplasmic scaffold protein DVL, of which three variants are known in humans (DVL1, 2 and 3) (Gao et al.,

2010). The DVL protein is characterized by three major domains: an N-terminal DIX domain, a central PDZ domain, and a C-terminal DEP domain (Li et al., 1999; Wong et al., 2000). DVL acts downstream of FZD as a scaffold protein and various reports suggest that DVL activates specific signaling pathways via distinct domains. For instance, the DIX domain is crucial for β-catenin-dependent signaling (Penton et al., 2002; Yanagawa et al., 1995), the PDZ domain for β-catenin-dependent as well as for β-catenin-independent signaling (Ciani et al., 2007; Habas et al., 2003; Krylova et al., 2000; Penton et al., 2002), while the DEP domain is essential mainly in β-catenin-independent signaling e.g. in the functional regulation of small GTPases like Rho by activating Rac and JNK (Axelrod et al., 1998; Habas et al., 2003; Rosso et al., 2005). Moreover, the DEP domain together with a classical C-terminal motif were shown to be required for FZD binding and WNT-induced β-catenin activation necessary in cultured cells and *Xenopus* embryos (Tauriello et al., 2012). In addition, DVL is known to get phosphorylated in response to WNT and FZD binding, both *in vivo* and *in vitro*, a process reportedly depending on a functional DEP domain (Bernatik et al., 2011; Bryja et al., 2007b; Rothbächer et al., 1995; Tada et al., 2000). Despite much emphasis on the role of DVL in WNT/FZD signaling, detailed mechanistic information on the structural or functional interaction of DVL with FZDs, and in combination with or without G proteins, remains unknown. The fact that the interface for G protein-FZD interaction (Wess, 1998) topologically overlaps considerably with the region of FZD-DVL interaction (Tauriello et al., 2012; Wong et al., 2000) creates further confusion in the search for a model describing this multi-protein complex. Concerning the FZD/Ca^{2+} pathway, it is not certain whether DVL acts upstream (Sheldahl et al., 2003) or downstream (Katanaev et al., 2005) of heterotrimeric G proteins. One likely model explaining the G protein-DVL relationship is that of dividing the FZD-transduced signaling simultaneously into fast G protein-dependent (Ma et al., 2006) and slow DVL-dependent signaling (Bryja et al., 2007a; González-Sancho et al., 2004; Liu et al., 2005). Another, maybe more

unconventional theory is that of FZD indirectly activating G proteins via DVL, similar to the proposed link between FZD_4 and β-arrestin (Chen et al., 2003). Such a detour could explain many of the experimental difficulties we and others are facing in studying FZD-G protein interaction (see also Materials & Methods section).

1.2.3.2 FZD co-receptors

Several different co-receptors are known to interact with FZDs in various ways, among them are e.g. low density lipoprotein receptor-related protein (LRP) 5/6, related to receptor tyrosine kinase (RYK), and receptor tyrosine kinase (ROR) 1/2 (discussed in detail in (Schulte, 2010)). Out of these, LRP5 and LRP6 have been studied the most in regards to WNT/FZD signaling. LRP5/6 are transmembrane proteins with a single membrane spanning domain and are well known to hold essential roles in the signal transduction of β-catenin-dependent WNT signaling (Pinson et al., 2000). Numerous reports have confirmed that several different WNTs can bind to the N-terminus of LRP5/6 to initiate signaling by further binding to FZDs and thus activating downstream signaling events (He et al., 2004; Itasaki et al., 2003; Liu et al., 2003; Mao et al., 2001).

1.2.4 WNT/FZD signaling pathways

Historically, WNT/FZD signaling has been divided into two main pathways depending on the involvement of β-catenin (formally termed β-catenin-dependent or β-catenin-independent signaling pathways), a versatile transcriptional regulator. With increasing knowledge of the field, relying on β-catenin only as a default player in the pathway has proven too simple in many instances, and thus most commonly the various pathways involving WNT/FZD are being referred to by their main components involved.

The β-catenin-dependent signaling pathway has been extensively studied since its discovery in the 1990's with the help of various robust assays such as transformation of C75MG cells (Wong et al., 1994), transcriptional regulation

of target genes (e.g. TOPFlash assay (activation of the luciferase T-cell specific transcription factor/lymphoid enhancer factor (TCF/LEF)-driven reporter (Molenaar et al., 1996; Van de Wetering et al., 1996))), β-catenin stabilization assayed by immunoblotting or immunocytochemistry, phosphorylation of upstream effector proteins, or dorsal body axis formation in *Xenopus* embryos (Heasman et al., 1994; Schneider et al., 1996).

In the absence of WNTs, cytosolic β-catenin levels are kept low by continuous phosphorylation through casein-kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3), which form part of a multimeric destruction complex besides other proteins like axin and adenomatous polyposis coli (APC) (Klaus et al., 2008; MacDonald et al., 2009). Upon the presence of ligands, WNTs induce interaction of FZDs and the co-receptor LRP5/6 by direct interaction with both. This causes CK1 and GSK3 to be released from the destruction complex and phosphorylate LRP5/6. Subsequently, further redistribution of proteins leads to a stabilization of β-catenin in the cytoplasm and translocation to the nucleus, where it binds TCF/LEF transcription factor family proteins to induce transcription of target genes related to e.g. cell proliferation, growth, and other vital cellular functions (Figure 1 top left two cartoons) (reviewed in detail in (Schulte, 2010) as well as http://wnt.stanford.edu).

Besides signaling to β-catenin, WNT/FZD signaling is involved in many other cellular pathways involving various proteins. Amongst the best described are: WNT/RAC, WNT/RHO, WNT/Ca^{2+}, FZD/PCP, WNT/cAMP, and WNT/ROR (Figure 1) (compare (Schulte, 2010; Semenov et al., 2007). Historically, with limited knowledge about the ever-growing complexity we have of WNT/FZD signaling today, it came easy to strictly divide the different pathways based on the involvement of β-catenin or major other players mentioned above. Technical advancements in biomedical sciences such as low-cost RNA/DNA sequencing (reviewed in (Goodwin et al., 2016)) or fast and relatively inexpensive gene editing tools such as CRISPR/Cas9 lead to an explosion of

discoveries in fields such as cell and molecular biology (Cong et al., 2013; Jinek et al., 2012). More and more evidence indicates that some of these previously strictly separated WNT/FZD pathways might after all converge, overlap or interact with each other at various points in a downstream signaling cascade (Halleskog et al., 2013; Park et al., 2015) (paper III).

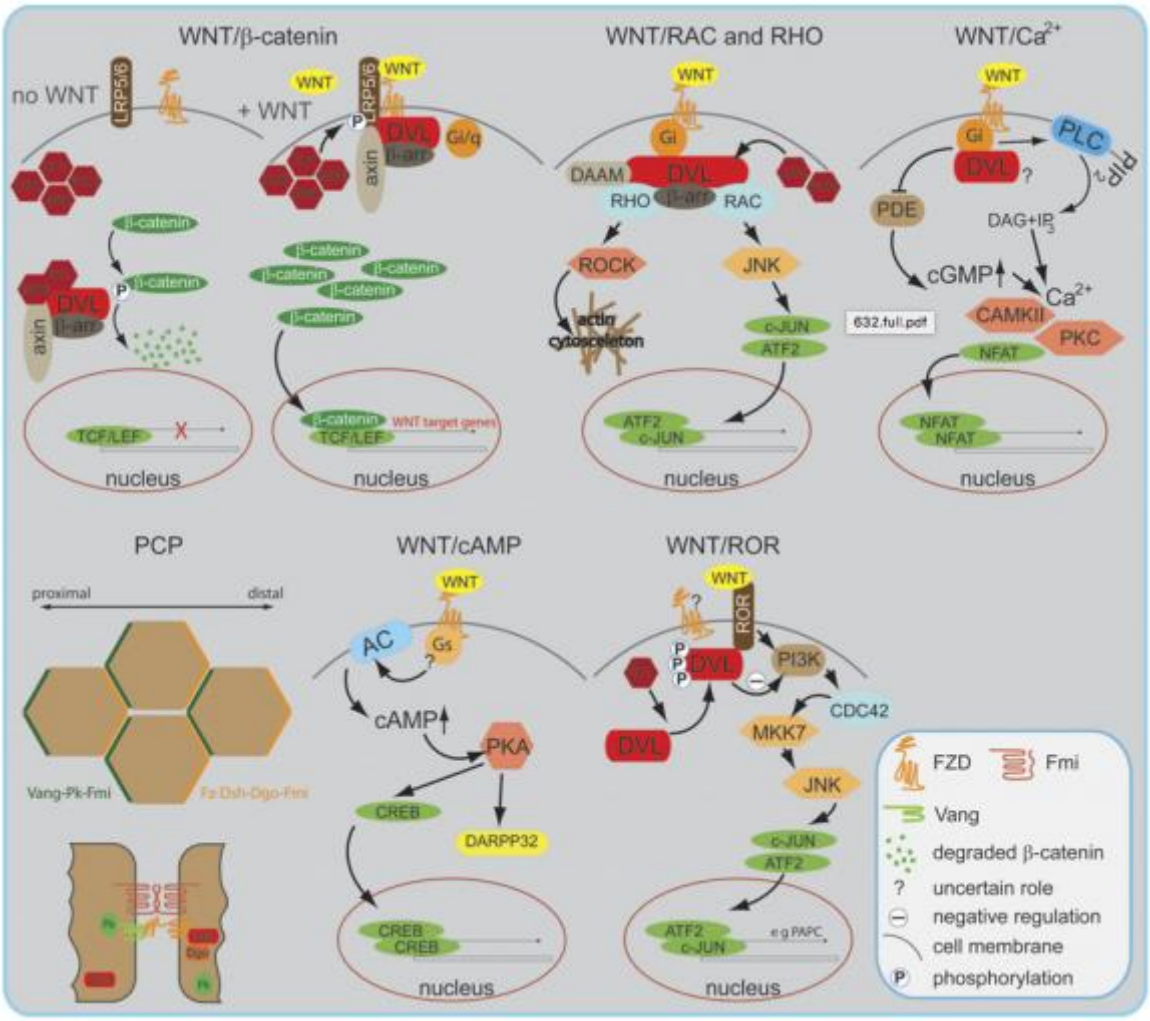

Figure 1. Schematic overview of WNT/FZD signaling. Individual cartoons depicting WNT/β-catenin, WNT/RAC and RHO, WNT/Ca^{2+}, PCP, WNT/cAMP, and WNT/ROR signaling pathways. Image from Schulte et al. 2010.

1.3 GPCR SIGNALING

The family of GPCRs comprises close to 800 members in the human genome and constitutes the largest, and at the same time, most diverse group of membrane receptors in eukaryotes (Bjarnadottir et al., 2006). GPCRs share common structural features including an extracellular N-terminus, a

hydrophobic 7-transmembrane-spanning core (7-TM), and an intracellular C-terminus. These cell surface receptors regulate a vast pool of cellular functions, and it comes as no surprise that roughly 30-40% of all approved drugs worldwide target members of this class of proteins (yet only roughly 50 GPCRs make up this vast number of drugs) (Hopkins et al., 2002; Wise et al., 2002). Interestingly enough, there are still about 120 members for which specific ligands have yet to be identified (Chung et al., 2008). As the name implies, GPCRs relay signals into the cell by means of heterotrimeric G proteins. Binding of an extracellular ligand to a GPCR induces a conformational change and causes subsequent heterotrimeric G protein recruitment to the receptor, accompanied by an exchange of GDP to GTP at the alpha subunit of the G protein, indicating the/accompanied by the activation of the G protein (Alexander et al., 2015) (for a comprehensive and up-to-date review see (Ghosh et al., 2015)).

A longstanding and still mostly valid model for the functional interaction of ligand, GPCR and heterotrimeric G proteins is the so-called “ternary complex model”, explaining the reversible interaction between a ligand, receptor, and G proteins (De Lean et al., 1980). Two main models have emerged over time and based on evidence, aiming at explaining the dynamics of the receptor-G protein interaction. The first model is called “collision coupling”, in which ligand-bound , and thus active, receptors and G proteins randomly collide and couple transiently and reversibly, resulting in the activation of the G protein (Gilman, 1987; Hein et al., 2009; Leff, 1995; Tolkovsky et al., 1978; Tolkovsky et al., 1981). This model confirms the original GPCR-G protein model hypothesizing that agonist binding is required for a GPCR-G protein physical interaction and activation to take place. Moreover, this model is also in agreement to some degree with the ternary complex model, where the agonist–receptor–G protein complex is stable without the presence of guanine nucleotides, and only once GTP exchange occurs the complex dissociates. The second model postulates

that GPCRs and G proteins can exist in a "pre-coupled" or "pre-assembly" state before agonist binding (Gales et al., 2006; Hein et al., 2009; Neubig, 1994; Rebois et al., 2003). Here, agonist binding initiates receptor activation, leading to conformational changes of the pre-assembled receptor-G protein complex and resulting in G protein. This model provides ideas for potentially accelerating the rate of G protein activation by eliminating the rate-limiting step of the collision coupling model, the random collision of receptor and G protein.

1.3.1 G proteins and FDZs

Soon after FZDs were first discovered, analyses of their primary amino acid sequence quickly led to the conclusion that they were, at least structurally, related to GPCRs (Barnes et al., 1998). Since then, an overwhelming amount of evidence in various organisms has provided compelling arguments for a FZD/G protein liaison (Arthofer et al., 2016; Egger-Adam et al., 2008; Kilander et al., 2014a; Kilander et al., 2011a; Kilander et al., 2014b; Riobo et al., 2007; Slusarski et al., 1997a; Slusarski et al., 1997b; Wu et al., 2000). Despite all the evidence of FZDs acting as "real" GPCRs, it cannot be denied that FZDs, being able to evidently signal without the involvement of heterotrimeric G proteins, are atypical GPCRs (Egger-Adam et al., 2008; Schulte et al., 2007). Recent studies from our group have shed new light on the involvement of DVL in regards to FZD-G protein signaling. On one hand, we observed that the interaction and signaling capacity of FZD_6 with $G\alpha_{i1}$ and $G\alpha_q$ depend on defined intracellular levels of DVL (Kilander et al., 2014b). In contrast, the inactive-state complex between FZD_4 and $G\alpha_{12/13}$ did not depend on DVL levels or its general presence at all (Arthofer et al., 2016), raising doubts whether a uniform FZD-G-protein-DVL complex model might even exist, as compared to a unique model for each FZD isoform-specific signaling route. Similarly, we recently generated data showing a FZD_4-$G\alpha_{12/13}$ inactive state complex assembly, which can be dissociated upon Norrin stimulation, irrespectively of pretreatment with Dickkopf-1 (DKK-1), an inhibitor of LRP5/6 binding to FZD, or co-

transfection of a dominant-negative phosphorylation-dead LRP6 mutant (Wolf et al., 2008). Phosphorylation of LRP5/6 by either GSK3 (Zeng et al., 2008; Zeng et al., 2005) or CK1 (Davidson et al., 2005; Zeng et al., 2005) is associated with the accumulation of proteins such as LRP6, Axin, and DVL, and viewed as a starting step for the formation of a LRP6 signalosome, causing β-catenin accumulation in the cytoplasm and activation of downstream WNT signaling.

One of the ultimate quests in cellular biology and signaling is to precisely define cellular circumstances leading to and executing specific signaling events. However, a multitude of factors such as type of ligands, co-factors, as well as co-receptors present, or cell type, cell compartment, and stage of development, or other yet unknown elements can all play a role in any given signaling event. Yet, in regards to WNT/FZD signaling, it becomes more and more obvious that there might be certain elements, which may dictate FZD isoforms to signal either towards heterotrimeric G proteins or signaling routes involving e.g. LRP6 and DVL.

1.4 GLIOMA MICROENVIRONMENT

Malignant gliomas are amongst the deadliest types of cancer and represent over 80% of malignant brain tumors. Glioblastoma is the most common malignant glioma subtype, accounting for almost 45% of all gliomas. The median survival time for patients suffering from glioblastoma is less than 15 months, with a 5-year relative survival of under 5% (Ostrom et al., 2014). The standard treatment regimen for malignant gliomas typically involves surgical resection followed by chemotherapy and/or radiation therapy (Stupp et al., 2007). The World Health Organization (WHO) has established a grading system, grade I-IV, for gliomas based on malignant behavior. The most commonly occurring histologic types of gliomas in adults, named after their cellular origin, include astrocytoma, oligodendroglioma, and oligoastrocytoma. Glioblastoma, also sometimes

referred to as glioblastoma multiforme (GBM) are classified as grade IV astrocytomas (Kleihues et al., 1993).

Gliomas are histologically very heterogeneous, which has shown to be one of the major obstacles for successful therapeutic intervention (Holland, 2000). It is clear today that the tumor microenvironment of malignant gliomas does not only significantly contribute to tumor initiation and progression, but also heavily to tumor metastasis (Hu et al., 2008). The complex glioma microenvironment is typically composed of neurons, astrocytes, microglia, fibroblasts, pericytes, and endothelial cells (Charles et al., 2011). Nonmalignant astrocytes are specialized glial cells in the brain providing structural support amongst other essential functions and have long been suspected to play a role in the progression of malignant brain tumors. Once attracted to the tumor, these tumor-associated astrocytes (TAAs) can become activated by various factors in the tumor microenvironment and display distinct epithelial-to-mesenchymal (EMT) transition and enhanced migration and invasion activity, making them one potential target for therapy (Charles et al., 2011; Lu et al., 2016; Seike et al., 2011; Shabtay-Orbach et al., 2015). In addition to astrocytes, microglia, the immunocompetent macrophages of the central nervous system (CNS), hold another important role in the pathobiology of malignant glioma. As the resident macrophages of the CNS, microglia have been shown to maintain homeostasis and respond to damages of the CNS with a range of reactions: change of morphology (“active” microglia) or motility, or changes in gene expression and pro-inflammatory cytokine release (Charles et al., 2011; Eggen et al., 2013; Gertig et al., 2014; Hanisch et al., 2007; Kettenmann et al., 2011). Microglia can be stimulated by various signaling molecules such as neurotransmitters, growth factors, or morphogens (Hanisch, 2002; Pocock et al., 2007), which emphasizes the importance of ongoing communication between microglia and e.g. astrocytes, neurons, or oligodendrocytes in determining microglia fate and functions in the CNS. Microglia activation is known to be important in

developmental processes in the brain like synaptic remodeling, but also accompanies several neurological pathologies such as infections, brain tumors, neuropathic pain, or neurodegenerative diseases (Graeber, 2010; Halleskog et al., 2011). Considering the enormous importance of both β-catenin-dependent and β-catenin-independent WNT signaling in neuronal development and homeostasis (De Ferrari et al., 2006; Inestrosa et al., 2010) (Henríquez et al., 2012), it comes as no surprise that WNT signaling can also have an effect on the microglia response in the brain (Halleskog et al., 2011; Kilander et al., 2011b; Pukrop et al., 2010).

Despite the role of microglia as the healthy brain's immune defense, various reports have shown that in glioma patients, microglia might have the potential to do the contrary, to promote tumor growth and migration (Bettinger et al., 2002; Markovic et al., 2005; Zhai et al., 2011). This is especially astonishing in the light of scientific evidence describing microglia as the antigen presenters of the CNS due to their expression of major histocompatibility (MHC) class II molecules (Proescholdt et al., 2001; Tran et al., 1998). While it seems that these cells are perfectly equipped to function as antigen presenting cells, functional antigen presentation to helper or cytotoxic T cells does not occur, leaving room for speculation as to what their real function is in malignant gliomas. One study explains this failed antigen presentation by pointing to a lack of expression of costimulatory factors and thus downregulation of T cell activation (Flügel et al., 1999).

1.4.1 Malignant gliomas and WNT signaling

Limited evidence is available thus far on the exact role WNTs play in malignant glioma pathophysiology. Some reports give importance to β-catenin-dependent signaling since high DVL2 levels in cultured and patient-derived glioma cells promote proliferation and differentiation, and likewise, cells with depleted DVL2 levels do not form tumors after intracranial injection into

immunodeficient mice (Pulvirenti et al., 2011). Furthermore, inhibition of GSK3β prevents neurosphere formation in ex vivo GBM cultures (Korur et al., 2009). Regarding the importance of specific WNTs in glioma pathophysiology, WNT-5A, previously shown to have both tumor suppressive and oncogenic effects in cancer (McDonald et al., 2009), has drawn the most attention thus far. First indications for a role of WNT-5A in malignant glioma came when several groups reported that Wnt5A levels in human GBM were higher compared to normal brain tissue and low-grade astrocytoma (Kamino et al., 2011; Pu et al., 2009; Yu et al., 2007) (Paper I). In addition, complementary in vitro studies in one of the recently-turned-infamous GBM cell lines U87MG (Allen et al., 2016) showed increased proliferation upon WNT-5A overexpression. Further studies complemented these initial findings confirming WNT-5A to be essential for glioma cell proliferation and invasion (Kamino et al., 2011; Pu et al., 2009; Pulvirenti et al., 2011). Despite several reports pinpointing WNT-5A to GBM progression, more research will need to be done to understand underlying mechanistic details.

1.4.2 WNT/G protein signaling and microglia

The idea of WNT signaling to heterotrimeric G proteins in microglia is rather young and was first formed when our group previously published findings that recombinant WNT stimulation activates heterotrimeric G proteins in primary mouse microglia cells and N13 microglia cells (Halleskog et al., 2012; Kilander et al., 2011a). In one study, they showed that recombinant WNT-5A provoked a proliferative response in the mouse microglia-like cell line N13, which was sensitive to PTX. In addition, activation of $G\alpha_{i/o}$ proteins in N13 and primary microglia by WNT-5A was corroborated using the GTPγS binding (Kilander et al., 2011a). In a further study, our group investigated intracellular transduction pathways in primary mouse microglia cells and revealed that WNT-5A activates heterotrimeric $G\alpha_{i/o}$ proteins to reduce cyclic AMP (cAMP) levels and to activate a $G\alpha_{i/o}$ protein/phospholipase C/calcium-dependent protein

kinase/ERK1/2 axis. In addition, they showed that ERK1/2 signaling induced by WNT-5A causes distinct aspects of the pro-inflammatory transformation seen in microglia, including proliferation, expression of matrix metalloprotease 9/13, invasion (Halleskog et al., 2012).

2 AIMS

This thesis aims to understand if, how, and where FZDs function as GPCRs and more precisely, to gain insight into mechanistic details of FZDs acting as GPCRs relevant for pathological manifestations. Specifically, our aims were:

- ◊ To investigate the presence of WNT-5A in glioblastoma samples and to decipher the role of WNT-5A within the glioma tumor microenvironment with a focus on tumor-associated microglia (Paper I).
- ◊ To explore G protein coupling and potential G protein specificity of FZD_4 (Paper II + III).
- ◊ To verify signaling activation through FZD_4 and $G\alpha_{12/13}$ induced by WNTs and Norrin (Paper II + III).
- ◊ To investigate Norrin-induced signaling via β-catenin-independent but G protein-dependent pathways (Paper III).

3 MATERIALS & METHODS

Most of the techniques used in the studies for this thesis are widely considered standard methods. For a detailed description of the procedures please see the Materials & Methods section in each individual publication. In this section, I will mainly describe the shortcomings of some or total lack of assays to study various aspects of WNT/FZD signaling.

Table 1. List of methods used in the publications comprising this thesis.

Method	Paper
Cell culture	II, III
Cell transfection	II, III
Molecular cloning	II, III
WNT/Norrin stimulation or inhibitor treatment	II, III
SDS-PAGE/Immunoblotting	II, III
Immunohistochemistry	I
Immunocytochemistry	II, III
Luciferase reporter assay	II, III
p115RhoGEF recruitment assay	II
Fluorescent/Confocal microscopy/Live cell imaging	I-III
Double color fluorescence recovery after photobleaching (FRAP)	II
Förster resonance energy transfer–Photoacceptor bleaching (FRET)	II
Bioinformatic analyses: TCGA GBM dataset analysis, MiMi interactome network analysis, MCODE analysis, GO analysis, GSEA analysis, KEGG pathway analysis, co-occurrence analysis	I
Dynamic mass redistribution	II

3.1 METHODOLOGICAL CONSIDERATIONS

3.1.1 Cell systems to study WNT/FZD signaling

One major challenged we face when studying WNT/FZD signaling on a molecular level is the choice of an appropriate cell model. Many commonly used and commercially available cell lines such as HEK293, HeLa, HEPG2, or others show expression of most of the 10 FZD isoforms (unpublished data and (Halleskog et al., 2012). Many of the 19 WNTs, which are all commercially available but often have doubtful purity and activity, have been shown to bind to, interact with, and act on more than one FZD isoform, creating numerous possible WNT/FZD interaction pairs (for an overview of reported WNT/FZD interactions see Figure 2 in (Dijksterhuis et al., 2014)).

Our laboratory recently published work on a 32D cell-based system engineered to overexpress FZD_2, FZD_4, or FZD_5, which allows for systematic assessment of functional selectivity of purified WNTs for individual FZDs. This is possible because 32D cells show low or undetectable endogenous mRNA levels for the ten FZD isoforms (Dijksterhuis et al., 2015b). Establishing FZD isoform-selective cell lines provides a substantial advantage for selectively targeting individual FZDs. On the other hand, using cells that endogenously express single FZD isoforms at probably sub-physiologically relevant levels could hint that those cells might not have all the necessary additional machinery for functional WNT/FZD signaling and thus are not ideal for studying physiological aspects of the WNT/FZD signaling pathway.

Equally challenging is the fact that almost all cells naturally express and secrete WNT proteins in an autocrine and paracrine fashion, which has been known across species for quite some time (Lawrence et al., 1996; Nolo et al., 2000; Zecca et al., 1996). The development of a class of pharmacological small-molecule WNT inhibitors called porcupine inhibitors, named after their inhibitory function of the membrane bound O-acyltransferase PORCN, which is required for WNT palmitoylation, secretion, and biologic activity (Kurayoshi et

al., 2007; Najdi et al., 2012; Proffitt et al., 2012; Takada et al., 2006). Currently, there are several inhibitors on the market available to use for biomedical research, which are C59 (Proffitt et al., 2013), LGK974 (Liu et al., 2013), ETC-159 (Madan et al., 2016), as well as inhibitors of WNT production (IWP)-2, -L6, -12 (Chen et al., 2009), some of which are currently also tested in clinical trials to inhibit aberrant WNT signaling (LGK974: NCT01351103, NCT02649530, NCT02278133; ETC-159: NCT02521844).

Another way to selectively study one FZD isoform only is by creating stable cell lines overexpressing a fluorescently-tagged or epitope-tagged FZD (unpublished data). This way, the FZD in question is stable (over)expressed and easily recognized by fluorescent microscopy or immunoblotting, and any pharmacological intervention such as ligand stimulation or inhibition is likely acting on the overexpressed FZD isoform and not on lower expressed endogenous FZDs. Nevertheless, said endogenous FZD expression or overexpression artefacts due to high exogenous protein levels in the cell can skew experiments performed with such cells. Therefore, any meaningful conclusions drawn with such overexpression systems need careful validation by employing several different experimental assays together to individually reach the same conclusions.

3.1.2 FZD and G protein activation assays

As mentioned before, even though FZDs are classified and widely acknowledged throughout the scientific community to be GPCRs, the main feature of GPCRs, which is to bind to and activate heterotrimeric G proteins, presents literally as “the struggle of a lifetime” for many researchers in the WNT field. Because of a wide array of potential WNT/FZD interactions and a lack of physiological cell models expressing a single FZD isoform only, studying G protein interaction and activation for individual FZDs has proven challenging. Radiometric methods such as radioligand binding or GTPγS

measurement were the first to be developed for GPCR activation, but are difficult to perform because they require advanced laboratory setups and again, endogenous FZD levels might skew any results obtained. Classical non-radiometric assays such as cAMP or calcium measurements, however, can only be used for selected Gα subtypes. For example, calcium measurement is selective for $G\alpha_q$-coupled receptors whereas cAMP is selective only for $G\alpha_s$ and $G\alpha_{i/o}$-coupled GPCR (see Table 2). Therefore, these assays require well-characterized signaling pathways, a fact which is near impossible in cell lines with more than one FZD expressed. In addition, several G protein activation assays known to work with a broad range of classical GPCRs turned out to not be suitable for studying FZDs (unpublished data), in many cases for unknown reasons, yet reinforcing the notion that class Frizzled receptors differ to varying degrees from classical GPCRs in function as well as G protein specificity and selectivity (Schulte, 2010).

Table 2. Summary of conventional G-protein dependent assays for mammalian cell systems suitable to study FZD-G-protein interaction with a focus on G alpha subunits. Additional information on G protein-specific pharmacological agents including references: PTX inhibits $G\alpha_{i/o/t}$ activation by catalyzing ADP-ribosylation of said G proteins (Barbieri et al., 1988; Bokoch et al., 1983; Casey et al., 1989); YM-254890 inhibits $G\alpha_q$ by inhibiting the GDP to GTP exchange (Taniguchi et al., 2003); UBO-QIC inhibits $G\alpha_{\mathbf{q}}$ by preventing the activation of $G\alpha_{\mathbf{q}}$ (Takasaki et al., 2004); GP-2A is a competitive $G\alpha_q$ inhibitor (Tanski et al., 2004); Y-27632 inhibits $G\alpha_{12/13}$ by specifically blocking RhoA (Ishizaki et al., 2000); Melittin inhibits $G\alpha_{\mathbf{s}}$ and activates $G\alpha_i$ (Fukushima et al., 1998; Raghuraman et al., 2007); The suramin analogue NF023 inhibits $G\alpha_{i/o}$ (Freissmuth et al., 1996); Green font indicates activation of the Gα subunit as opposed to black font indicating inhibition.

G protein family	α subunit	Main signal transduction route	Commonly available assays	G protein-specific pharmacological intervention	Evidence for G protein-FZD interaction
G_i family					
$G_{i/o}$	α_i, α_o	Inhibition of adenylyl cyclase (⇓ cAMP)	cAMP assay Ca^{2+} GTPγS binding SRE reporter	Pertussis toxin (PTX) Suramin analogue NF023 Melittin ($G\alpha_i$)	$rFZD_2$ ($G\alpha_i$ or $G\alpha_o$) - (Slusarski et al., 1997a) $FZD_?$ ($G\alpha_o$) – (Liu et al., 2005) $hFZD_1$ ($G\alpha_o$) – (Katanaev et al., 2009) $hFZD_1$ ($G\alpha_i$) – (Koval et al., 2011) $hFZD_6$ ($G\alpha_i$) –(Kilander et al., 2014b)
G_t	α_t (Transducin)	Activation of phosphodiesterase (PDE)	GTPγS binding Time-resolved fluorescence spectroscopy		$rFZD_6$ ($G\alpha_t$) - (Ahumada et al., 2002)
G_{gust}	α_{gust} (Gustducin)	Activation of PDE	Ca^{2+} cAMP		
G_z	α_z	Inhibition of adenylyl cyclase (⇓ cAMP)	cAMP		
G_s family					
G_s	α_s	Activation of adenylyl cyclase (⇑ cAMP)	cAMP FZD Co-IP CRE reporter	Melittin Cholera toxin ($G\alpha_s$)	FZD_7 – (von Maltzahn et al., 2011)
G_{olf}	α_{olf}	Activation of adenylyl cyclase (⇑ cAMP)	cAMP CRE reporter		
G_q family					
G_q	α_q, α_{11}, α_{14}, α_{15}, α_{16}	Activation of phospholipase C (⇑ IP_3 and Ca^{2+})	Ca^{2+} IP_3 NFAT-RE reporter	YM-254890 ($G\alpha_q$) FR900359/UBO-QIC ($G\alpha_q$) GP-2A ($G\alpha_q$)	$rFZD_2$ ($G\alpha_q$) – (Ma et al., 2006) $hFZD_6$ ($G\alpha_q$) – (Kilander et al., 2014b)
$G_{12/13}$ family					
$G_{12/13}$	α_{12}, α_{13}	Activation of the Rho family of GTPases Ectodomain shedding of TGFα	p115-RhoGEF recruitment SRF-RE reporter Yap/Taz reporter TGFα shedding	C3 toxin (blocks Rho)	FZD_1 ($G\alpha_{12/13}$) – (Park et al., 2015) $hFZD_4$ ($G\alpha_{12/13}$) – (Arthofer et al., 2016) $hFZD_{10}$ ($G\alpha_{13}$) – (Hot et al., 2017)

4 RESULTS & DISCUSSION

Ever since the discovery of FZDs and the subsequent notion that this class of receptors shares key structural features with the superfamily of GPCRs, their potential to function as GPCRs has been disputed. Based on overwhelming structural and functional evidence in various species over the past decades, FZDs were grouped into their own class F among the GPCR family by the IUPHAR and The British Pharmacological Society in "The Guide to Pharmacology" (Alexander et al., 2015; Foord et al., 2005; Schulte, 2010). Despite a growing pool of evidence showing direct interactions of FZDs and heterotrimeric G proteins (see Table 2 and recently reviewed in (Dijksterhuis et al., 2014)), we and many other groups studying WNT/FZD signaling pathways remain largely in the dark when it comes to describing details of structural, functional, and physiological properties of WNTs, FZDs, G proteins and other involved components of this signaling path/of WNT/FZD signaling mechanistics.

4.1 WNT-5A IN HUMAN MALIGNANT GLIOMA

In order to answer the ever-present questions, if, how, and where FZDs function as GPCRs, we had previously been studying WNT signaling in microglia cells. By using immortalized N13 and primary mouse microglia cells we could show that microglia are sensitive to ectopic WNT-3A and WNT-5A stimulation by increasing their proliferative potential, which for most part could be blocked by the $G\alpha_{i/o}$ inhibitor PTX (Halleskog et al., 2012; Halleskog et al., 2013; Kilander et al., 2011a; Kilander et al., 2011b). WNT-5A stimulation of microglia also mirrored increased invasive potential and caused upregulation of inflammatory markers by these cells (Halleskog et al., 2012). Since microglia represent the immunocompetent cells of the brain and CNS and play an important role in brain inflammatory processes, we decided to turn to brain tumors to investigate the role of WNT signaling and WNT-5A further.

4.1.1 High levels of WNT-5A in human glioma correlate with increased presence of tumor-associated microglia

Based on several reports showing upregulation of WNT-5A in human glioma and GBM samples compared to healthy brain samples as well as in glioma-derived cell lines (Kamino et al., 2011; Pu et al., 2009; Yu et al., 2007), we set out to confirm these findings and investigate further. In paper I, we found that in a tissue microarray with 48 malignant gliomas and 40 healthy brain samples WNT-5A was significantly upregulated in the tumor tissue when analyzed via immunohistochemistry. These results were supported by a meta-analysis of the TCGA-GBM dataset, showing >4-fold upregulation of WNT-5A in GBM samples compared to healthy brain. Despite this, there was no significant difference on the median survival of patients showing high WNT-5A mRNA levels, pointing towards a more complex effect of WNTs on the pathobiology in malignant glioma.

Nevertheless, we further compared mRNA expression levels of two groups of patients, the 25% of patients with highest and lowest WNT-5A expression ($WNT\text{-}5A^{high}$ vs $WNT\text{-}5A^{low}$). We found that high WNT-5A levels correlated with high expression of genes associated with biological processes linked to immunological responses as defined by standard Gene Ontology terminology eg. immune response (GO:0006955) and antigen processing and presentation of peptide or polysaccharide antigen via MHC class II (GO:0002504) (Figure 2). Surprised by the novelty of those results, we performed further in-silico analyses using bioinformatics tools such as gene set enrichment analysis (GSEA), the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009a; Huang et al., 2009b), or the software Cytoscape in combination with additional plugins such as MiMI or MCode (http://allegroviva.com/allegromcode) (Gao et al., 2009; Saito et al., 2012). These results also pointed towards a connection between high WNT-5A levels and upregulation of genes involved in immune responses and antigen

processing. This is in line with the fact that GBMs are known to create local and systemic immunosuppression by e.g. regulating cytokine expression in the tumor microenvironment thus inducing immune tolerance in the pathologic brain (Gomez et al., 2006; Hussain et al., 2006; Komohara et al., 2008; Prosniak et al., 2013; Reardon et al., 2014; Szulzewsky et al., 2015; Waziri, 2010).

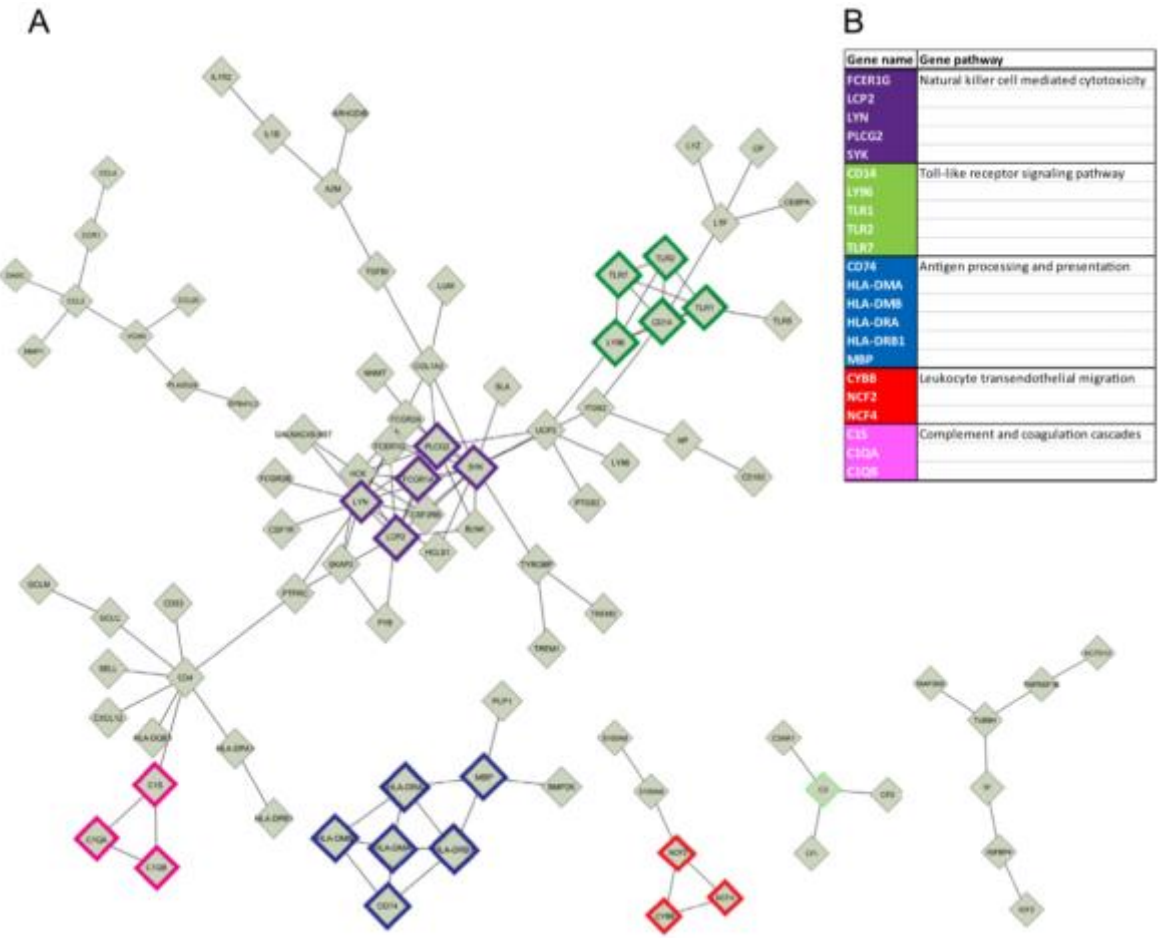

Figure 2. High WNT-5A levels in human GBM correlate with high expression of a network of genes linked to immunological processes. (A) MiMI interactome network (Michigan Molecular Interactions plugin for Cytoscape) visualizing gene-gene interaction networks was defined by determining the differentially expressed genes between the WNT-5A^{low} versus WNT-5A^{high} subgroups of the TCGA GBM sample set available at https://cancergenome.nih.gov/cancersselected/glioblastomamultiforme. Networks with N≤3 genes were excluded (B) MCODE analysis of the MiMI interactome network with corresponding color coding in (A) finds clusters in a network representing densely connected regions. Image from (Dijksterhuis et al., 2015a)

Besides a correlative indication for an immune-response signature in the WNT-5A^{high} group, we also found enrichment of known microglia marker genes (Kettenmann et al., 2011) in this patient subgroup. Co-occurrence analysis between high WNT-5A and microglia markers confirmed a positive correlation between these two, providing hints for the origin of the immune response seen on mRNA levels. Furthermore, additional analyses of the TMAs revealed co-localization of the microglia marker ionized calcium-binding adapter molecule 1 (IBA-1) and HLA-DMA/HLA-DPB1, two major molecules involved in MHC class II antigen processing and response. This co-localization showed significant correlation (Pearson coefficient of 0.6144 (p=0.0014) for IBA-1/HLA-DMA and 0.4458 (p=0.0290) for IBA-1/HLA-DPB1), indicating a biologically relevant and statistically significant relationship between HLA components and microglia infiltration in the glioma tissue tested. These results are in line with the fact that microglia are antigen presenting cells and reports pointing to microglia as the source of inflammation in brain tumors and neurodegenerative diseases. Subsequent analysis of other MHC class II pathway components revealed that several, but not all, important members were indeed also enriched in the WNT-5A^{high} group. The fact that not all necessary components of the MHC class II pathway were present in the glioma samples analyzed is partly in line with reports showing impaired MHC class II antigen presentation in cells of the tumor microenvironment and could be one mechanism how cancer cells avoid host immune responses (Schartner et al., 2005). This finding, together with the idea that high levels of microglia infiltration into tumor tissues are associated with a poor disease prognosis (Kuang et al., 2007; Yang et al., 2010), could to some extent explain the aggressiveness and poor prognostic outcome of malignant gliomas. In addition, considering studies our group published previously on the effects of WNT-5A on microglia migration, invasion, and pro-inflammatory transformation, events, which were PTX-sensitive and thus $G\alpha_{i/o}$-mediated (Halleskog et al., 2012; Kilander et al., 2011a), it is reasonable to assume that most likely some of the

WNT-5A-correlative features observed in GBM in paper I might also be heterotrimeric G protein-dependent.

In summary, even though from our data in paper I we cannot directly explain the effect WNT-5A exerts on glioma progression, our results point towards a novel role of WNT-5A in relation to malignant glioma-associated microglia infiltration and possibly also local immunosuppression. Further studies outlining more phenotypical details of the tumor microenvironment and immunological processes underlying glioma progression are needed to evaluate whether elevated microglia presence caused by WNT-5A in these tumors might present a valid target for drug development.

4.2 FZD_4-G PROTEIN INTERACTION

In our quest to dissect WNT/FZD signaling and investigate the mechanisms of FZD-G protein interaction, we made use of an established imaging method for determining lateral mobility of receptor and G protein after immobilization of a distinct receptor fraction and subsequent stimulation of the receptor. This live-cell imaging approach, FRAP, has been used successfully to study the interaction of cytokine receptors and kinases (Giese et al., 2003), interaction details of receptors and ion channels (Lober et al., 2006), dissociation of G protein subunits (Digby et al., 2006), analysis of receptor oligomerization (Dorsch et al., 2009), or inactive-state complex formation of muscarinic receptors and heterotrimeric G proteins (Qin et al., 2011). One should mention here that when we employ dcFRAP in our studies, we investigate FZD and G protein interaction relating to the proposed "preassembly" or "inactive state complex" model, similar to what has been described for the M_3 receptor using the same methodology (Qin et al., 2011). This model for receptor-G protein interaction studied here should not be mistaken with the well described receptor-G protein coupling, which describes a model for an agonist-activated receptor-G protein complex, as was exemplified first with the β_2-adrenergic receptor in complex with $G\alpha_s$ (Rasmussen et al., 2007).

4.2.1 A FZD_4-$G\alpha_{12/13}$ inactive state complex dissociates upon WNT stimulation and assembles independently of DVL

In paper II, we set out to provide functional evidence strengthening the idea that FZDs function as GPCRs (Foord et al., 2005; Kilander et al., 2014b). By using a double-color fluorescence recovery after photobleaching (dcFRAP) technique, we were able to investigate the inactive-state assembly of receptor and G protein in question. FRAP allowed to distinguish whether FZD_4 and members of the four families of heterotrimeric G proteins are assembled or separated in the membrane at any given time. This method is based on immobilization of transmembrane proteins by chemical surface crosslinking (CL) with sulfo-NHS-LC-LC-biotin and avidin. Contrary to transmembrane receptors, intracellular proteins, such as heterotrimeric G proteins, are not directly affected by surface CL in this assay and their mobile fraction is reduced only upon interaction with CL-immobilized surface proteins, such as transmembrane receptors. Using this approach in an overexpression system with HEK293 cells, we could show for the first time in mammalian cells that FZD_4 forms an inactive-state complex with $G\alpha_{12/13}$ (Figure 3), but not with any of the other family members like $G\alpha_{i/o}$, $G\alpha_s$, or $G\alpha_q$. Adequate controls like using a no-receptor control or a non-FZD GPCR control allow us to determine with very high confidence that the observed decrease in lateral mobility of the G protein in question is indeed caused by the formation of an inactive-state assembly with the FZD isoform studied. Furthermore, this complex dissociated upon WNT stimulation, in the dcFRAP assay, in FRET (Förster resonance energy transfer) measurements, as well as indirectly using dynamic mass redistribution (DMR), which allows label-free measuring of ligand-induced changes in living cells (Grundmann et al., 2015; Schröder et al., 2011).

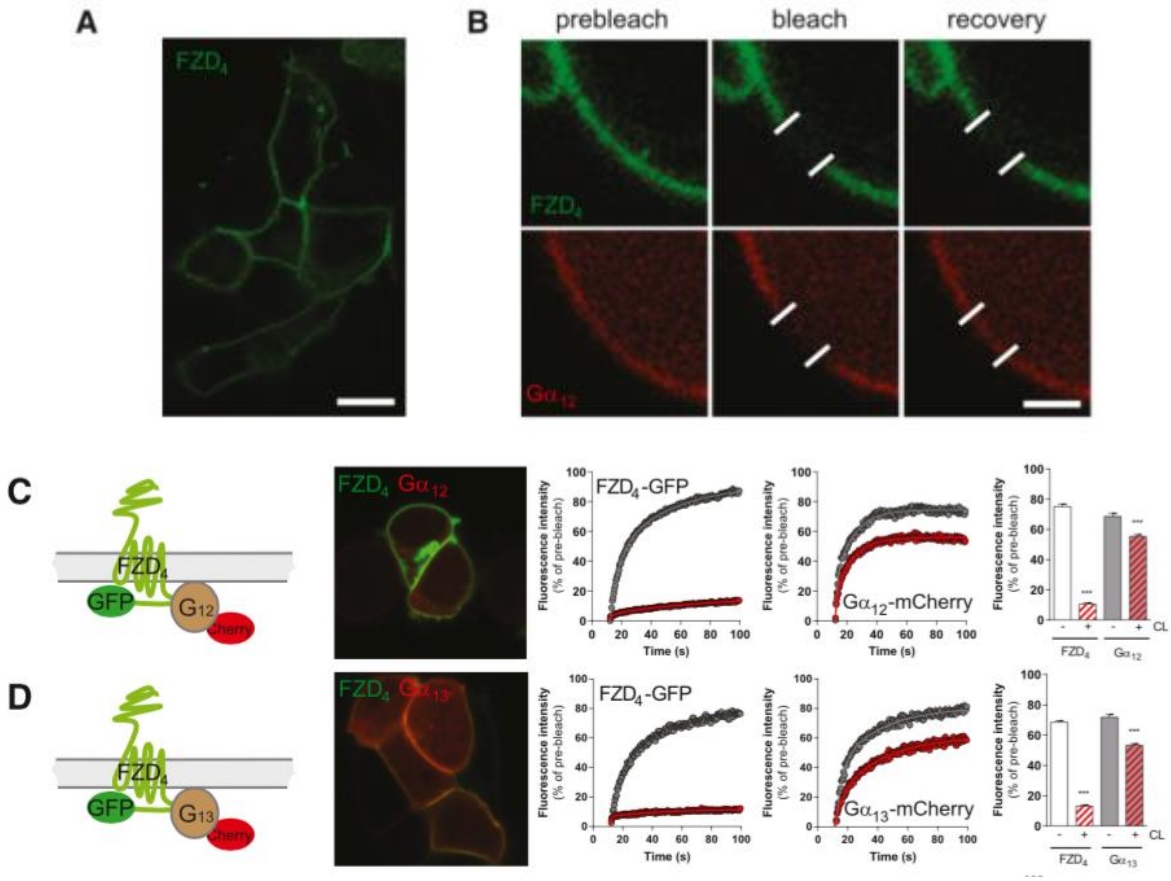

Figure 3. FZD$_4$ and Gα$_{12/13}$ form an inactive-state assembly complex in HEK293 cells. (A) Fluorescently-tagged hFZD$_4$ is expressed primarily in the plasma membrane of HEK293 cells. (B) Sketches representing various time points as indicated during the dcFRAP analysis. White bars indicate the selected area in the plasma membrane that got bleached with high laser intensity. (C-D) Schematic presentation clarifying the experimental setup, a confocal micrograph showing HEK293 cells transiently expressing FZD$_4$ with the respective Gα subunit, fluorescence intensity curves before (gray) and after (red) CL for both FZD$_4$ and the respective G protein and a bar graph summarizing the mobile fractions of FZD$_4$ and the Gα subunit under each experimental condition. Color code for mobile fractions: white, FZD$_4$ before CL; red hatched, FZD$_4$ after CL; gray, Gα before CL; gray + red hatched, Gα after CL. ***p<0.001 (n = 3). Error bars provide the S.E.M. ns = not significant.

Altogether, our data showing the dissociation of the Gα subunits indicate a functional FZD$_4$-Gα$_{12/13}$ complex with the potential to initiate signaling through the dissociated G protein subunits.

In order to further describe this FZD$_4$-Gα$_{12/13}$ complex and understand how signals get relayed from outside the cell to the inside, we took a look at the scaffold protein DVL, an important co-factor of the WNT pathway. From studies with FZD$_6$ we knew that defined cellular levels of DVL were required

for functional assembly of FZD_6 with $G\alpha_i$ or $G\alpha_q$ (Kilander et al., 2014b). In regards to FZD_4-$G\alpha_{12/13}$, we determined that DVL was not only dispensable but excessive cellular levels also did not disturb the assembly of FZD_4 with $G\alpha_{12/13}$ (only increasing exogenous DVL levels to three times the amount commonly transfected for this protein prevented complex formation (unpublished data)).

4.2.2 A WNT-FZD_4-$G\alpha_{12/13}$-p115-RhoGEF signaling axis

Heterotrimeric $G\alpha_{12/13}$ signal predominantly to the Ras homology gene family, member A (RhoA) GTPase and induce downstream signaling effects in cells such as changes in cell shape, migration, and adhesion (Worzfeld et al., 2008). $G\alpha_{12/13}$ signaling to RhoA depends on RhoGTPase guanine nucleotide exchange factors (RhoGEFs) such as p115RhoGEF, which interacts with the active, GTP-bound and from βγ–subunits dissociated $G\alpha_{12/13}$ (Hart et al., 1998). In paper II, we show that FZD_4 induces p115RhoGEF recruitment to the plasma membrane (Figure 4), strongly arguing for the fact that FZD_4 is acting as a guanine nucleotide exchange factor (GEF) on heterotrimeric $G\alpha_{12/13}$ proteins.

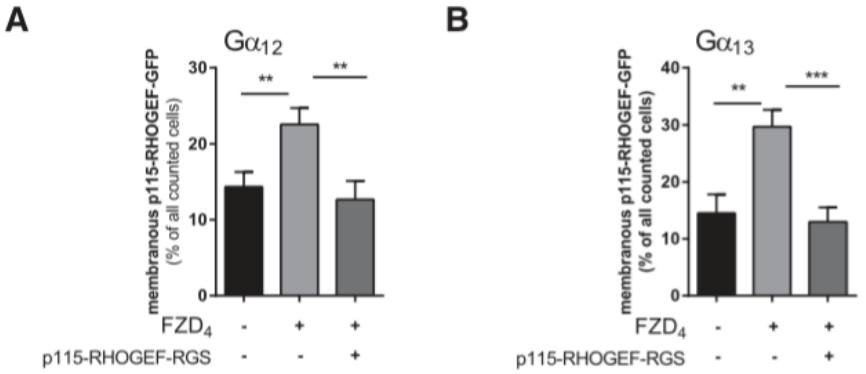

Figure 4. FZD_4 induces $G\alpha_{12/13}$-mediated p115-RhoGEF membrane recruitment, which is dependent on the activation of heterotrimeric $G\alpha_{12/13}$ proteins. HEK293 cells transiently transfected with vectors for FZD_4, $G\alpha_{12}$ (A) or $G\alpha_{13}$ (B), and p115-RhoGEF either without or with the isolated RGS domain of p115-RhoGEF-RGS. Cells presenting membranous p115-RhoGEF localization were counted manually and data from four independent experiments were summarized in the bar graph. Experiment contain at least 50 counted cells for each condition and in each independent experiment. Values given represent mean ± S.E.M.

Since we lack robust pharmacological tools for selectively targeting of FZD isoforms, we incubated with the porcupine inhibitor C59 in this assay, which has been shown to strongly reduce autocrine WNT secretion (Proffitt et al., 2013). This had a significant negative impact on the p115RhoGEF recruitment, confirming that FZD_4-$G\alpha_{12/13}$ signaling is WNT-dependent. Complementary to this, when we added the p115-RhoGEF RGS domain, which acts as a selective GTPase-activating protein (GAP) for $G\alpha_{12/13}$ (Hart et al., 1998; Kozasa, 1998) and promotes hydrolysis of GTP to GDP thus rendering the G protein inactive, we observed a reduction of the FZD_4-induced p115RhoGEF recruitment back to basal levels (Figure 4). Taken together, these data strongly support for the functionality of a WNT-FZD_4/$G\alpha_{12/13}$-p115-RhoGEF signaling axis with a dependence on FZD_4-mediated activation of $G\alpha_{12/13}$.

4.2.3 Norrin signaling through a FZD_4-$G\alpha_{12/13}$ signaling axis

In paper III, which is currently in manuscript form, we built on the findings from paper II, namely that FZD_4 forms an inactive-state complex with and activates $G\alpha_{12/13}$ and take them one step further. We show that the FZD_4-selective and WNT-unrelated protein ligand Norrin, which is considered a selective agonist for the FZD_4-LRP5/6 receptor complex, can also activate FZD_4-G protein signaling along the $G\alpha_{12/13}$ axis but independent of LRP5/6. These novel findings add to the complexity of the FZD-ligand repertoire and largely unknown selectivity, as Norrin has thus far only ever been reported to interact with FZD_4 together with the co-receptor LRP5/6 to activate WNT/β-catenin signaling in mammalian cells (Xu et al., 2004). Furthermore, we shed light on the interaction between the historically separated and generally termed β-catenin-dependent and β-catenin-independent WNT signaling pathways in regards to FZD_4 and thus offer hints that these pathways most likely share common elements or converge at various points to either potentiate or inhibit signaling outcomes.

Based on our previous findings of a FZD_4-$G\alpha_{12/13}$ signaling axis, we set out to test the non-WNT ligand Norrin and its ability to dissociate this receptor-G protein complex. In line with results we observed previously when stimulating with WNTs (paper II), the FZD_4-$G\alpha_{12/13}$ inactive-state complex dissociated upon Norrin stimulation in a dcFRAP setup in HEK293 cells, pinpointing to an active ligand-receptor-G protein complex. Because Norrin has in the past repeatedly been associated with FZD_4 and LRP5/6 (Junge et al., 2009; Xu et al., 2004), we investigated the importance of these FZD co-receptors by overexpressing a dominant negative and phosphorylation-deficient LRP6-AAAAA construct (Wolf et al., 2008). However, we did not observe any changes in the dcFRAP assay showing the formation of a FZD_4-$G\alpha_{12/13}$ complex in the presence of the dominant negative LRP6 construct. Furthermore, to verify the functionality of this complex and its independence of endogenous LRP5/6 we pre-incubated with the selective LRP5/6 inhibitor DKK-1, which interferes with LRP5/6 involvement, before stimulating with Norrin. Despite blocking LRP5/6, Norrin still caused dissociation of $G\alpha_{12}$ from FZD_4. These data suggest that Norrin is capable of activating FZD_4 without recruitment of LRP5/6. This is particularly interesting for two reasons: one, in light of previous reports of Norrin and FZD_4 claiming LRP5/6 as a crucial cofactor in this complex to initiate β-catenin-dependent signaling (Junge et al., 2009; Xu et al., 2004; Ye et al., 2009); two, underlying mutations for diseases such as familial exudative vitreoretinopathy (FEVR), an inherited blinding disorder of the retinal vascular system (Criswick et al., 1969), have initially been found in FZD_4 and Norrin, but later also in LRP5/6 (Chen et al., 1993; Jiao et al., 2004; Robitaille et al., 2002; Toomes et al., 2004).

To further investigate the activated signaling paths downstream of FZD_4 and $G\alpha_{12/13}$, we focused on recent reports claiming a connection between $G\alpha_{12/13}$ signaling and RHO activation feeding into YAP/TAZ signaling (Hot et al., 2017; Park et al., 2015; Schlessinger et al., 2009; Yu et al., 2012; Zhao et al.,

2012). To explore this hypothesis, we tested the synthetic 8xGTIIC luciferase reporter assay to monitor transcriptional activation downstream of the transcriptional regulators YAP and TAZ (Dupont et al., 2011). Overexpressing FZD_4 in HEK293 cells provoked a stable induction of the YAP/TAZ luciferase reporter, which was absent in HEK293 cells lacking both $G\alpha_{12}$ and $G\alpha_{13}$. Further, adding the $G\alpha_{12/13}$-selective regulator of G protein signaling (RGS) domain of p115RhoGEF, which functions as a GTPase-activating protein (GAP), completely abrogated both basal and FZD_4-induced YAP/TAZ activation in parental cells, but had little effect in $G\alpha_{12/13}$ KO cells. This confirms that the FZD_4-induced activation of YAP/TAZ is dependent on $G\alpha_{12/13}$. Based on the surprising findings that LRP5/6 was irrelevant for formation and dissociation of a FZD_4-$G\alpha_{12/13}$ inactive-state complex in HEK293 cells, we tested the role of LRP5/6 in regards to YAP/TAZ activation. When using the 8xGTIIC luciferase reporter in LRP5/6 deficient and corresponding parental HEK293 cells, FZD_4 expression induced stable YAP/TAZ-dependent transcriptional activity in parental cells and significantly higher activity in LRP5/6 deficient cells. These results corroborate the idea that a FZD_4-$G\alpha_{12/13}$-YAP/TAZ signaling route does not require LRP5/6, and might even be partly inhibited by it, and thus constitutes a parallel signaling path to the well-known Norrin-FZD_4-LRP5/6 signaling axis described above.

Stunned by the novel observation that FZD_4 has the potential to activate a $G\alpha_{12/13}$–mediated and YAP/TAZ-dependent signaling path, we wanted to know whether this $G\alpha_{12/13}$-dependent FZD_4 signaling axis might interfere with the known FZD_4-LRP5/6-β-catenin signaling route. To do this, we tested the β-catenin TOPFLash luciferase reporter in $G\alpha_{12/13}$ KO and corresponding parental cells in combination with FZD_4, Norrin, and LRP5/6. Surprisingly, the TOPFlash signal induced by FZD_4-Norrin-LRP5 or FZD_4-Norrin-LRP6 increased in the $G\alpha_{12/13}$ KO cells. This increase was partially reversed by expressing ectopic $G\alpha_{12}$ or $G\alpha_{13}$ proteins. Furthermore, expression of the RGS

domain of p115RhoGEF, which promotes GTP hydrolysis and thus inactivation of the G protein, enhanced the TOPFlash response in parental cells in combination with FZD_4-Norrin-LRP6. All things considered, the results strongly point towards active FZD_4-$G\alpha_{12/13}$ signaling as a negative regulator of FZD_4-β-catenin signaling. Based on our results obtained, we provide new evidence for a central role of $G\alpha_{12/13}$ in FZD_4-mediated signaling to β-catenin as well as β-catenin-independent downstream targets.

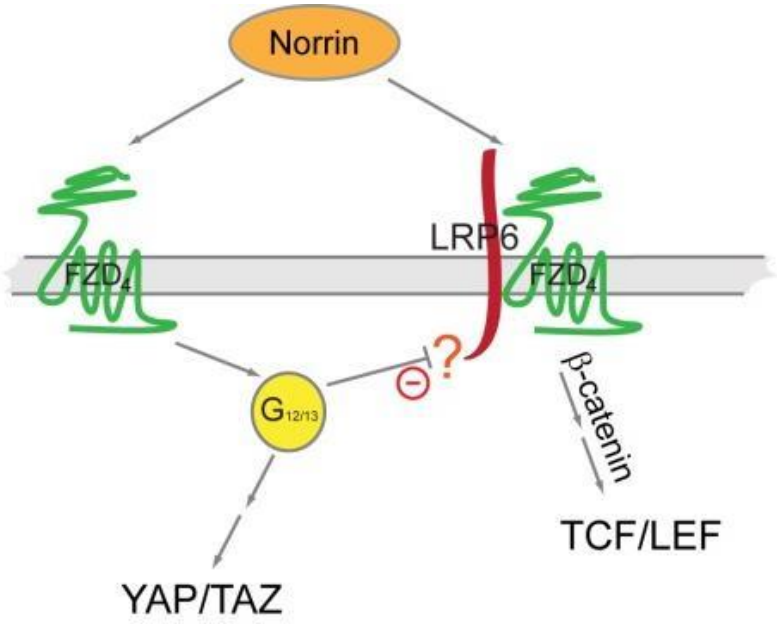

Figure 5. $G\alpha_{12/13}$ regulates a Norrin-FZD_4 signaling loop. Schematic cartoon of a proposed Norrin-FZD_4 signaling loop regulated by $G\alpha_{12/13}$. On one hand, Norrin binding to FZD_4, together with LRP5/6, activates β-catenin and TCF/LEF-mediated transcriptional regulation. On the other hand, Norrin binding to FZD_4 causes a dissociation of $G\alpha_{12/13}$ and induces YAP/TAZ-mediated transcriptional regulation sensitive to blockage through the RGS-domain of p115RhoGEF. The crosstalk level and details of the negative feedback of $G\alpha_{12/13}$ signaling towards the FZD_4-Norrin-LRP5/6-β-catenin axis remain vague.

On one hand, we show that Norrin can act as a FZD_4 agonist to induce LRP5/6-independent signaling. Regarding physiological functions involving FZD_4, this could mean that endothelial cell physiology in angiogenesis might not only

depend on the known FZD-LRP5/6-β-catenin signaling axis but also on a novel FZD_4-$G\alpha_{12/13}$ signaling route. Such a concept would be in line with findings suggesting a crucial role for $G\alpha_{12/13}$ in angiogenesis (Offermanns et al., 1997; Sivaraj et al., 2013). On the other hand, we provide hints for a concept that states that the FZD_4-β-catenin pathway involving LRP5/6 might ultimately be controlled and regulated by FZD_4-G protein signaling. Since both signaling routes seem to be present and activated in parallel, G protein-mediated inhibition could play an important role in FZD_4 signal compartmentation and bidirectional pathway regulation (Figure 5). Taken together, besides the known role of FZD_4 in β-catenin-dependent signaling (Junge et al., 2009; Xu et al., 2004; Ye et al., 2009), our evidence strongly suggests a β-catenin-independent role of FZD_4-$G\alpha_{12/13}$, not just in combination with Norrin (paper III) but also WNT stimulation (paper II).

5 GENERAL DISCUSSION & PROSPECTIVE OUTLOOK

WNT/FZD signaling has been studied extensively for over three decades and remains one of the most enigmatic distinct signaling pathways known in biology. Class Frizzled receptors share several typical features of classical GPCRs, yet detailed mechanistic descriptions are rare for the mere endless pool of signaling combinations of WNT-FZD-G protein and other co-factors in this pathway. In this thesis, I aimed to give insights into WNT/FZD pharmacology from various aspects, starting from biology and pathophysiology in regards to WNT-5A to signaling mechanistics of FZD_4. In the various studies discussed herein, we attempted to dissect the details lacking in the current understanding of WNT/FZD signaling cascades, from ligand binding to receptor and G protein involvement and activation to downstream signaling specification and signaling initiation resulting in cellular changes and potential feedback loops. This thesis provides novel elements on the previously unappreciated depth and complexity of WNT/FZD signaling and by no means intends to provide a complete story but rather to serve as the basis for further studies and for the development of more sophisticated tools and techniques to study WNT/FZD signaling in unprecedented detail.

Some of the results reported in this thesis raise various interesting questions providing grounds for discussions and further research (and more sophisticated methodological tools), some of which I would like to briefly address here. Generally, studying WNT/FZD pharmacology is cumbersome due to several factors:

i. *Receptor-ligand complexity:*

Enormous diversity of WNT signal activation exists due to the presence of 19 WNTs and 10 FZDs in mammals, as well as several co-receptors. When looking at research from our group and many others in the field, it has become more and more obvious that various FZDs can respond to the same WNT isoform in many of the conventional cell lines studied, and

most certainly also in the living organism. Therefore, precise receptor profiles and expression levels of the cell system in question are necessary in order to define a specific WNT-FZD target signaling route.

ii. Ligand intricacy:

WNT proteins are secreted molecules influencing and orchestrating a myriad of cellular processes. Due to the addition of lipid adducts during posttranslational modifications, WNTs are highly hydrophobic and tend to "stick" to a multitude of carrier molecules or the plasma membrane. Therefore, it is difficult to determine a physiological effective concentration of WNTs. In addition, due to the intrinsic instability of WNTs, knowing the exact concentration of a given recombinant WNT product is near impossible. Therefore, important pharmacologically terms such as efficacy, affinity, and potency are difficult to assess when dealing with recombinant WNTs.

iii. Class Frizzled receptors as unconventional GPCRs

Despite sharing several structural features of classical GPCRs, it remains a challenge to study FZD-G protein interaction, and so far, no attempt to obtaining a crystal structure of a FZD has been successful. Based on reports and studies performed in this thesis, it has become clear that FZDs can act as both classical and non-classical GPCRs, signaling via heterotrimeric G proteins or without the help thereof. These mechanistic subtleties may likely depend on the cell type or tissue in question and their repertoire at of endogenous WNTs, FZDs, and co-factors at any given time.

Considering the above-mentioned difficulties, it is obvious that classical tools for studying GPCR activation are most likely bound to fail. It cannot be emphasized enough, that studying WNT/FZD/G protein signaling pathways requires the use and development of alternative and sophisticated approaches,

likely with the need to tailor them based on the cell type or experimental model used. A start to this would be the much-needed development of isoform-selective small-molecule inhibitors for FZDs.

In the first part of this thesis, we tried to elucidate the presence and role of WNT-5A in GBM. WNT-5A has for quite some time been linked to cancer, but astonishingly, this WNT protein is capable of acting in an oncogenic as well as tumor-suppressing manner, depending on the cancer type (view (Asem et al., 2016) for a comprehensive and up-to-date review). Based on previous studies by our group and others, we knew that microglia play an important role in the immunological protection of the brain but also in GBM progression (Yang et al., 2010). At the same time, WNT-5A exerts proliferative effects on microglia (Kilander et al., 2011a), which are G protein-dependent, and can cause pro-inflammatory cytokine release (Halleskog et al., 2012) and increase migration and invasion of microglia (Halleskog et al., 2012). Several studies showing microglia are a major component of the glioma microenvironment as well as our findings on the effect of WNT-5A led us to investigate this WNT in GBM. As discussed for paper I, the involvement of WNT-5A in the overall GBM disease progression is not entirely clear. We can conclude that high WNT-5A mRNA levels result in high protein levels in the tumor microenvironment of GBM patients. In addition, in these WNT-5A^{high} patients increased numbers of microglia are found, yet no correlative effect on the overall survival was found. This raises doubts whether targeting WNT-5A pharmacologically in these patients might add any additional benefits. The second highlight of paper I, namely the finding that WNT-5A^{high} patients showed an increase in MHC Class II components, might present as a more attractive target. Targeting the increased number of microglia in the tumor microenvironment, or manipulating the tumor and its environment to express all necessary factors for functional MHC Class II antigen presentation might provide one option, yet certainly not a cure, for the devastating prognosis of this disease.

In the second part of this thesis, we aimed to more closely investigate the signaling specifics of the WNT receptor FZD_4. Even though this receptor has repeatedly been implicated in physiological processes, all mostly relating to angiogenesis (Robitaille et al., 2002; Xu et al., 2004; Ye et al., 2009) as well as some cancers (Acevedo et al., 2007; Planutis et al., 2007), few mechanistic details are known about FZD_4-specific signaling pathways. In paper II, we shed light into a major "black box" regarding FZD_4 signaling by defining the family of heterotrimeric G proteins this receptor binds to and interacts with, as $G\alpha_{12/13}$. With the help of a recently developed and successfully used approach to determine GPCR-G protein complex formation, double color FRAP (Dorsch et al., 2009; Giese et al., 2003; Qin et al., 2011), we could so far define G protein interactions for FZD_4 (Arthofer et al., 2016), FZD_6 (Kilander et al., 2014b), and FZD_{10} (Hot et al., 2017). Regarding the interaction of FZD_4 with $G\alpha_{12/13}$, these results might not come as a complete surprise considering that studies in the past described a crucial role for $G\alpha_{12/13}$ in angiogenesis (Offermanns et al., 1997; Sivaraj et al., 2013), aligning with the proposed physiological roles observed for FZD_4 in angiogenesis (Junge et al., 2009; Xu et al., 2004; Ye et al., 2009). Despite the almost obvious overlaps, no other studies so far have suggested a functional interaction between FZD_4 and $G\alpha_{12/13}$. In paper III, we further deepened our focus on the non-WNT and FZD_4-selective ligand Norrin. Building on the newly discovered connection between FZD_4 and $G\alpha_{12/13}$ as well as known reports of a Norrin-FZD_4-LRP5/6 signaling route towards β-catenin-mediated signaling, we established the existence of a Norrin-FZD_4-$G\alpha_{12/13}$ signaling axis, independent of LRP5/6 (and unfortunately thus yet another WNT/FZD signaling cascade). Further experiments revealed what recent reports have been suggesting too, which is that distinct FZD signaling paths, or simply β-catenin-dependent and β-catenin-independent pathways, might interact, share common features, or even potentiate or inhibit each other's signaling outcomes. It would add tremendous amount of knowledge to the field if detailed concepts of how one distinct FZD signaling route affects or interacts

with another distinct FZD signaling path. For reasons discussed above in i-iii as well as time and methodological limitations, more in-depth studies on these cross-pathway signaling relationships were not within the scope of this thesis.

In summary, the results presented and discussed in this thesis contribute to the understanding of WNT/FZD pharmacology and signaling. We aimed at investigating the WNT/FZD signaling pathway at different levels, ranging from basic pharmacological and biochemical properties to pathobiological relevance in brain tumors. The overall goal of our work is to improve the understanding of this important family of signal transduction pathways to accelerate the development of therapeutic approaches targeting this pathway in the future.

Ultimately, the objective of performing basic research is the idea to understand how the human body works and how to intervene when processes go wrong. This is, as many researchers know, much easier said than done. The majority of findings coming from basic research are likely to never get "translated" into any form of clinical use. Even research findings, which do end up going through some or all of the several stages of (pre)-clinical development, it is not ensured they will result in a medicinal product or medical device ultimately aiming to improving human health and wellbeing. In order to improve this, I am proposing several approaches for the future: modern concepts for biomedical research facilities should incorporate the idea of translational science being an integral part already at the level where basic science is performed. Second, improved and regular communication and scientific exchange between scientists of various areas, whether this might be basic sciences, applied sciences, high-throughput screenings and drug discovery, or in vivo research, should be of utmost importance to everyone involved in performing research in these areas. Incorporating broad knowledge and research findings from all these

disciplines, continuously and at an early stage in research, will be extremely beneficial for the advancement of biomedical research.

6 SUMMARY

Paper I

- WNT-5A protein levels are upregulated in human glioma tissue compared to non-malignant control brain tissue.
- WNT-5A is the only representative of the WNT ligand family upregulated on mRNA levels in human glioma samples compared to non-malignant control brain samples.
- High WNT-5A expression levels in gliomas are associated with upregulation of immune-related and pro-inflammatory factors.
- High levels of WNT-5A expression in gliomas correlate with a significant increase of HLA gene family-positive microglia/monocytes.
- Based on previously published in vitro data, the effects of WNT-5A on glioma-associated microglia could at least in part be G protein-dependent.

Paper II

- FZD_4 assembles with $G\alpha_{12}$ and $G\alpha_{13}$ to form an inactive-state complex in mammalian cells.
- The FZD_4-$G\alpha_{12/13}$ complex dissociates upon WNT stimulation as observed by dcFRAP, FRET, and indirectly also by DMR measurements.
- The WNT scaffold protein DVL is dispensable for FZD_4-$G\alpha_{12/13}$ complex formation.
- DMR measurements show that WNT-induced changes are $G\alpha_{12/13}$-dependent, in HEK293 cells overexpressing FZD_4 as well as at endogenous levels.
- FZD_4-$G\alpha_{12/13}$ mediate RHO signaling through membrane recruitment of p115-RhoGEF.

Paper III

- FZD_4-$G\alpha_{12/13}$ complex formation does neither depend on the functionality nor activation of LRP5/6.
- Norrin stimulation dissociates and activates a FZD_4-$G\alpha_{12/13}$ inactive state complex, independent of LRP5/6, pointing towards a previously unknown and β-catenin-independent Norrin-FZD_4-$G\alpha_{12/13}$ signaling route.
- FZD_4 mediates YAP/TAZ signaling in a $G\alpha_{12/13}$-dependent manner and independently of LRP5/6, thus representing a parallel signaling path to the recognized Norrin-FZD_4-LRP5/6 signaling axis.

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